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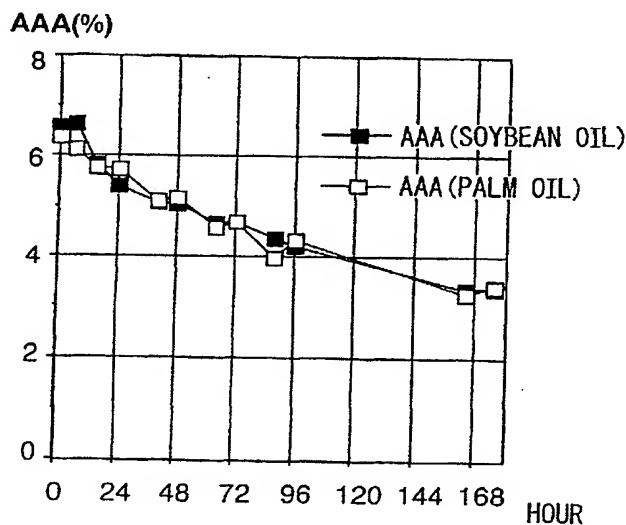
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(54) Title: PROCESS FOR PRODUCTION OF TRANSESTERIFIED OILS/FATS OR TRIGLYCERIDES



(57) Abstract: There are provided polyunsaturated fatty acids-containing oils/fats or triglycerides with high digestion and absorption properties and resistance to oxidative damage, which are suitable for applications in fields such as modified milk for infants, food products and healthy foods and/or supplements, produced by transesterification of polyunsaturated fatty acids-containing oils/fats or triglycerides with vegetable oils/fats or triglycerides using 1,3-position specific type lipases.

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## DESCRIPTION

PROCESS FOR PRODUCTION OF TRANSESTERIFIED  
OILS/FATS OR TRIGLYCERIDES

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## TECHNICAL FIELD

The present invention relates to a method of improving microbe-produced oils/fats or triglycerides which comprise polyunsaturated fatty acids as constituent fatty acids, by lipase transesterification, as well as to improved oils/fats or triglycerides and to human nutritive compositions comprising the improved oils/fats.

## BACKGROUND ART

15 Eicosapentaenoic acid (hereinafter referred to as "EPA") and docosahexaenoic acid (hereinafter referred to as "DHA") are the polyunsaturated fatty acids known in particular for their numerous physiological functions, including preventive effects against adult diseases such as arteriosclerosis and thrombosis, anticancer effects and learning reinforcement effects, and they are often utilized in drugs and special healthy foods and/or supplements. Recently, however, the physiological functions of other polyunsaturated fatty acids have been the subject of increasing attention.

20 Arachidonic acid is one of these polyunsaturated fatty acids and constitutes approximately 10% of the fatty acids composing important organs such as the blood and liver (for example, the compositional ratio of fatty acids in human blood phospholipids is 11% arachidonic acid, 1% eicosapentaenoic acid and 3% docosahexaenoic acid). As a major constituent of cell membranes it is involved in regulating membrane fluidity, and performs various roles in biometabolism while also serving as an important direct precursor to prostaglandins. A recent area of attention has been the role of arachidonic acid as an infant nutrient and its presence as a constituent

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fatty acid of endogenous cannabinoids (2-arachidonoyl monoglycerol, anandamide) which exhibit a neurostimulating action. Consumption of linoleic acid-rich foods usually results in conversion to arachidonic acid, but direct ingestion of arachidonic acid in the form of triglycerides is preferred because of a reduced function of the enzymes involved in biosynthesis in adult disease patients and those at risk, infants and the elderly who, as a result, tend to be deficient in arachidonic acid.

Fish oil is an abundant source of EPA and DHA, but dihomo- $\gamma$ -linolenic acid, arachidonic acid and 4,7,10,13,16-docosapentaenoic acid (22:5  $\omega$ 6) are almost unobtainable from conventional oil and fat sources. At the current time, it is common to use oils/fats or triglycerides whose constituent fatty acids are polyunsaturated fatty acids and which are obtained by fermentation with microbes. For example, according to one proposed method, various microbes capable of producing oils/fats or triglycerides containing arachidonic acid as the constituent fatty acid are cultured to yield the oils/fats or triglycerides containing arachidonic acid as the constituent fatty acid. Such methods include those for obtaining arachidonic acid-rich oils/fats or triglycerides using fungi of the genus *Mortierella* (see, for example, Japanese Unexamined Patent Publication No. 63-44891 and Japanese Unexamined Patent Publication No. 63-12290). Triglycerides containing fermentation-produced arachidonic acid as the constituent fatty acid are used for purposes requiring arachidonic acid, such as in the field of infant nutrition and, particularly, in modified milk.

Methods of adding arachidonic acid-containing oils or fats to modified milk have been disclosed in the field of infant nutrition (see Japanese Unexamined Patent

Publication No. 11-151075, Japanese Unexamined Patent  
Publication No. 10-191886). The arachidonic acid-  
containing oils or fats used as additives are produced by  
fungi, and from a molecular standpoint are characterized  
by comprising 6-24% AAA (a triglyceride with 3 residues  
of arachidonic acid in the molecule). A higher  
arachidonic acid content is known to result in a higher  
AAA concentration (see, for example, Jim-Wen Liu et al.,  
In vitro hydrolysis of fungal oils: distribution of  
arachidonic acid-containing triacylglycerol molecular  
species, J. Am. Oil Chem. Soc., 75, pp.507-510(1998)).

Oils and fats containing a high concentration of AAA  
differ from vegetable oils and fats in being resistant to  
the action of human digestive enzymes (pancreatic  
lipases) under physiological conditions and, therefore,  
such oils and fats are not readily digested and absorbed  
by infants or elderly with low pancreatic lipase activity  
(see, for example, Jim-Wen Liu et al.).

As the arachidonic acid content of human breast milk  
is 0.5% of the total fatty acids (see, for example,  
Christie, W.W., "The positional distribution of fatty  
acids in triglycerides" in Analysis of oils and fats,  
Edited by Hamilton, R.J. and Russell, J.B., pp.313-339,  
Elsevier Applied Science, London (1986)), presumably  
there is a higher probability of one arachidonic acid  
residue per triglyceride molecule rather than greater  
arachidonic acid condensation per molecule as in AAA  
described above. Consequently, addition of arachidonic  
acid-containing oils and fats obtained by fermentation  
with fungi to modified milk simply on the basis of the  
fatty acid content is not desirable.

Many attempts have been made to enzymatically alter  
oils and fats to enhance their properties (solubility in  
the mouth, crystallinity, heat resistance). Most have  
involved enzymatic modification of vegetable oils or  
fats, such as enzymatic synthesis of cacao substitute  
oils (see, for example, Japanese Unexamined Patent

Publication No. 55-71797, Japanese Unexamined Patent Publication No. 58-42697). The production technology disclosed here is for vegetable oils and fats that exhibit high lipase reactivity, whereas oils and fats  
5 rich in polyunsaturated fatty acids with poor reactivity are poorly suitable in terms of lipase reactivity.

Oils/fats or triglycerides rich in polyunsaturated fatty acids have not been used for enzymatic modification because of their poor enzyme reactivity, but it has been  
10 attempted to produce lipids with polyunsaturated fatty acids structure using medium-chain fatty acid- and polyunsaturated fatty acids-rich oils and fats, by using altered immobilized enzymes (see Japanese Unexamined Patent Publication No. 8-214891, PCT/JP02-06702).  
15 According to this method, the fatty acid at the 1- and/or 3-positions of the triglyceride are replaced with octanoic acid, thereby releasing the free polyunsaturated fatty acids. Polyunsaturated fatty acids-rich oils or fats are therefore not expected products. Another  
20 disadvantage is that further purification techniques such as precision distillation and the like are required to remove the free fatty acids.

Fujimoto et al. have studied in detail the effects of oil/fat structure on the oxidative stability of  
25 polyunsaturated fatty acids, in light of the fact that fish oils (particularly oils rich in EPA and DHA) readily oxidize and adequate oxidative stability cannot be achieved with addition of antioxidants (see, for example, Fujimoto, K., "Effects of oil and fat structure on  
30 oxidation stability of polyunsaturated fatty acids", Science and Industry, 75, pp.53-60, Osaka Industrial Research Association (2001)). An improvement in oxidative stability was discovered by adding special fats (C8 and C14 triglycerides of medium chain fatty acids) to  
35 sardine oil (rich in polyunsaturated fatty acids) and conducting transesterification with position non-specific type lipases, and upon examining the ease of oxidation of

the chemically synthesized polyunsaturated fatty acids triglyceride EEE (tri-EPA), and comparing types with the polyunsaturated fatty acids dispersed among the molecules and with a higher condensation in the same molecule, the dispersed type was confirmed to be satisfactory.

Transesterification has been disclosed as a method of stabilizing oils and fats in order to prevent oxidation of polyunsaturated fatty acids in fish oils and the like (see Japanese Unexamined Patent Publication No. 6-287593). However, the polyunsaturated fatty acids in fish oils and the like have low reactivity for the lipases used in transesterification reactions. According to this method, therefore, vegetable oils or fats with high lipase reactivity are used in a large amount to dilute one or more polyunsaturated fatty acids-containing oils purified from fish oil or the like in order to ensure lipase reactivity. As a result, it is not possible to achieve an increased content of polyunsaturated fatty acids in the stabilized oils and fats.

#### DISCLOSURE OF INVENTION

It has therefore been a desired goal to provide oils/fats or triglycerides containing polyunsaturated fatty acids which can be applied in the fields of modified milk for infants, food products and healthy foods and/or supplements, and which exhibit properties such as ready digestion and absorption and resistance to oxidative damage. In order to achieve this goal, the present invention provides a method of improving oils/fats or triglycerides containing polyunsaturated fatty acids such as arachidonic acid by lipase transesterification reaction in order to produce oils and fats with dispersed polyunsaturated fatty acids, as well as compositions comprising them and food products or healthy foods and/or supplements containing them.

Polyunsaturated fatty acids-rich oils/fats or

triglycerides having high polyunsaturated fatty acids contents and exhibiting high digestion and absorption properties as well as resistance to oxidative damage were successfully obtained by transesterification of 50-100 parts by weight of one or more oils/fats or triglycerides containing at least 20% of fungus-produced polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds and 0-50 parts by weight of one or more vegetable oils/fats or triglycerides, in a deoxygenated state using a 1,3-position specific type lipase, with an increased reaction temperature.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the effects of palm oil and soybean oil on reduction of triarachidonic acid triglyceride (AAA) in a transesterification reaction.

#### DETAILED DESCRIPTION OF THE INVENTION

Specifically, the present invention provides a method suitable for commercial production of oils and fats converted by transesterification reaction, which has been accomplished by:

discovering a method whereby an immobilized enzyme may be used to increase thermal stability and allow use of the enzyme at a high reaction temperature, while oxygen in the immobilized enzyme itself may be removed to avoid oxidative damage of the raw material/reacting oil during the reaction at high temperature, thus preventing oxidative damage and accomplishing a practical enzyme reaction; and

discovering a method whereby moisture used for activation of the enzyme may be efficiently eliminated after activation of the enzyme to minimize the amount of free fatty acids, monoglycerides and diglycerides which are secondarily produced as the transesterification reaction occurs with an equilibrium between hydrolysis and ester synthesis reactions, thus accomplishing a

practical enzyme reaction.

The invention therefore provides a method for producing edible transesterified oils and fats obtained by lipase transesterification reaction using fungus-produced polyunsaturated fatty acids-containing oils/fats or triglycerides as raw materials, as well as compositions containing the oils and fats.

Since the arachidonic acid content of breast milk lipid is less than 1 wt%, it is assumed that at most only one arachidonic acid residue is present in each triglyceride molecule. The molecular species in an arachidonic acid-rich oils/fats produced by fermentation of a fungus belonging to the genus *Mortierella* (SUNTGA40S: trade name of Suntory Co., Ltd.) are listed in Table 1.

Table 1 Forms of arachidonic acid in SUNTGA40S

Molecular species	Content (wt%)
AAA	10.57
XAA	37.56
XXA	26.52
XXX	25.35

Here, A represents arachidonic acid bound to the triglyceride, and X represents a fatty acid other than arachidonic acid bound to the triglyceride. Specifically, "AAA" represents a triglyceride with 3 arachidonic acid residues in the molecule, "XAA" represents a triglyceride with 2 arachidonic acid residues and a residue of a fatty acid other than arachidonic acid, and "XXA" represents a triglyceride with 1 arachidonic acid residue and two residues of a fatty acid other than arachidonic acid. Triglycerides containing arachidonic acid constitute 74.7% of the total triglycerides, but this consisted of 26.52% XXA which is presumed to be present in breast milk, and 48% of AAA or XAA which are believed to be not present. It is thought that a form of arachidonic acid close to that in breast

milk can be obtained by converting the 48% of AAA and XAA to the form of XXA.

5 The present inventors attempted to produce transesterified oils and fats with relatively low amounts of vegetable oils and fats using 1,3-position specific type lipases in a deoxygenated state for the purpose of improving the aforementioned fungal arachidonic acid-rich oils/fats and triglycerides produced by fermentation. As a result the inventors succeeded in dispersing  
10 arachidonic acid bound at 1- and 3-positions of a raw material AAA triglyceride which is abundant in fungal arachidonic acid oils and fats and is not digested or absorbed, while also approximating the structural form of human breast milk (XXA).

15 The oils and fats modified by the present transesterification method may be utilized for a wide range of purposes including addition to modified milks in the field of infant nutrition, as well as for food products and healthy foods and/or supplements.  
20 Methods of enzyme activation and oxygen removal

Methods of improving oils and fats by enzymatic transesterification have long been implemented and are described in prior art documents. The present arachidonic acid-rich oils and fats include many  
25 arachidonic acid residues such as AAA which do not readily function under physiological lipase reaction conditions, and this has constituted a problem for enzyme reactivity in transesterification. For increased transesterification efficiency it is desirable to improve  
30 the ester-exchange rate by immobilizing the lipase and conferring heat resistance for an increased reaction temperature (see, for example, Japanese Unexamined Patent Publication No. 8-214891, PCT/JP02-06702). However, when the reaction is conducted at high temperature to improve  
35 the reaction efficiency, the arachidonic acid-rich oils or fats as the raw material tends to undergo oxidative damage. Particularly when an immobilized enzyme is used,

a problem is posed by removal of oxygen which has penetrated into the immobilizing resin. The present inventors therefore discovered a method for efficiently removing oxygen from the immobilized enzyme and developed a method for transesterification under low-oxygen conditions.

The POV (peroxide value) is widely used as a parameter indicating the degree of oxidative damage of oils or fats. The amount of oxygen in the reaction system was varied for the transesterification reaction and the degree of oxidative damage of the oils or fats was compared by measuring the POV. The amount of oxygen was adjusted on the following 4 levels: Highest deoxygenation level = "deairing and nitrogen blow", Level with nitrogen substitution by ordinary nitrogen blow = "nitrogen blow", Level with absolutely no nitrogen substitution of reaction vessel = "no nitrogen substitution", Final use of only an immobilized enzyme support for control to a non-nitrogen substituted condition = "controlled". The POV values were measured after reaction for a prescribed period at temperatures of 30°C, 45°C and 55°C. As shown in Table 2, the POV rises in direct proportion to the amount of residual oxygen and, particularly, as the POV rose with increasing reaction temperature in the "nitrogen blow" deairing condition at the level with nitrogen substitution by ordinary nitrogen blow, removal of oxygen was thought to be insufficient. This means that reaction under this condition cannot prevent deterioration of the oils or fats, and these results revealed that the POV increase can be suppressed by increasing the deoxygenation to the next level of "deairing and nitrogen blow" to obtain oils or fats of sufficient quality.

Table 2 POV with reaction under different conditions

	30°C, 1 day	45°C, 3 days	55°C, 1 day
Deairing and nitrogen blow	0.87	0.88	0.87
Nitrogen blow	1.07	1.23	2.75
No nitrogen substitution	1.28	1.86	3.15
Controlled	2.69	4.50	4.05

(POV: meq/kg oil/fat)

In the case of *Rhizopus delemar* (current name:  
 5 *Rhizopus oryzae*) lipase, a small amount of moisture  
 (approximately 2%) must be added for enzyme activation.  
 This necessitates a procedure for eliminating the excess  
 moisture in the activated immobilized enzyme. This  
 procedure must also be accompanied by a procedure for  
 10 deoxygenation, which can be carried out simultaneously  
 with the enzyme activation or moisture elimination. At  
 the enzyme activation stage, the oil or fat raw material  
 and a small amount of water are added to the immobilized  
 enzyme and reaction is conducted at 30-45°C for about one  
 15 day for enzyme activation. For simple activation of the  
 immobilized enzyme, an inexpensive vegetable oil or fat  
 may be used instead of an expensive polyunsaturated fatty  
 acids-containing oil or fat. The oxygen removal may be  
 accomplished either during or before the stage of adding  
 20 the moisture-containing oil or fat raw material to the  
 immobilized enzyme, using a vacuum device such as a  
 vacuum pump for deairing of the oil or fat raw material  
 and immobilized enzyme, and allowing leakage of nitrogen  
 gas instead of air when the pressure is restored to  
 25 ordinary pressure. The oxygen in the immobilized enzyme  
 can be removed out by repeating this vacuum/pressure  
 restoration procedure. After completion of the  
 activation reaction, the oil or fat is removed, and the  
 oxygen is eliminated from the oil/fat-absorbed  
 30 immobilized enzyme by further repeating the  
 aforementioned vacuum/nitrogen gas leakage procedure.  
 The oxygen-eliminated (nitrogen-substituted) oil/fat raw

material is then added for transesterification, which can be accomplished while preventing oxidative damage of the oil or fat even under high temperature conditions.

5 Method of eliminating moisture used for enzyme activation or eliminating enzyme present in enzyme

The excess moisture must be eliminated from the immobilized enzyme activated in the aforementioned procedure. The presence of excess moisture promotes hydrolysis by lipases and results in secondary production of diglycerides, monoglycerides and free fatty acids during the transesterification reaction. It is necessary to remove the moisture from the reaction system to prevent such production. The excess moisture can be eliminated by removing the oil/fat used for activation from the activated immobilized enzyme, washing once with the oil/fat of the reaction raw material (oil or fat which has been already deoxygenated or dehydrated) and then reacting it with the crude oil/fat at 45°C for one day. This subsequent reaction yields a transesterified oil or fat with a low amount of diglycerides, etc. If the purpose is to lower the production cost, an economical vegetable oil or fat such as palm oil may be used instead of an expensive polyunsaturated fatty acids-containing oil or fat for the activation and moisture-eliminating reaction.

While enzyme activation with water has been necessary in the case of immobilizing enzymes of *R. delemar*, a commercially available immobilized enzyme (the *Rhizomucor miehei* lipase Chirazyme L-9, c.-f., C2, lyo, product of Berlinger Mannheim) requires no activation with water but is in an active state due to the trace amount of water present in the immobilized enzyme. However, excess moisture is also included in this immobilized enzyme, and therefore the moisture must be eliminated as in the case of *R. delemar*. The method used for moisture elimination may be the one described above,

and no special procedures are required. The elimination of oxygen may also be carried out in the dehydration step for this enzyme as well.

#### 5 Reactor for enzyme reaction

The enzyme reaction has been explained above based on transesterification in a batch system.

10 A column system is an alternative to a batch system as the method for transesterification of one or more oils or fats using an immobilized enzyme. Specifically, the immobilized enzyme is packed into a column-type jacketed container, the oil or fat raw material is introduced into the column while incubating or heating to adjust the column temperature, and the transesterified oil/fat is  
15 taken out from the outlet. In some cases, the oil/fat may be circulated while being passed through the column to increase the conversion rate. Here, the oil/fat raw material may be passed through the column during activation or moisture elimination to expose the  
20 immobilized enzyme in the interior to the oil, in combination with vacuum deairing and forced nitrogen gas blow to eliminate the oxygen. The oil or fat may be transesterified after activation and removal of the moisture.

25

#### Oil/fat raw material for transesterification

When one or more oils/fats or triglycerides comprising polyunsaturated fatty acids as a constituent fatty acid are mixed at 50-100 parts by weight with 0-50  
30 parts by weight of one or more vegetable oils/fats or triglycerides, a homogeneous liquid oil or fat is obtained and transesterification with an oxygen-eliminated immobilized enzyme as has been hitherto described may be carried out to obtain a modified oil or  
35 fat containing polyunsaturated fatty acids.

<Vegetable oils/fats>

The vegetable oil or fat raw material used for the reaction may be one commonly used in foods. The vegetable oils and fats used may also be converted oils and fats. Such vegetable oils and fats are used as sources of saturated fatty acids for transesterification. As examples there may be mentioned linolic acid-rich oils such as soybean oil, safflower oil, olive oil, rice bran oil, sesame oil and the like, oleic/linolic acid-rich oils and saturated fatty acid-rich oils such as palm oil, palm kernel oil, cacao oil, coconut oil, lard oil and the like, and oils obtained by conversion from triglyceride vegetable oils such as MCTs (triglycerides comprising saturated medium-chain fatty acids of 8 carbons and saturated medium-chain fatty acids of 10 carbons, and composed of the same or different fatty acids). Saturated fatty acid-rich oils and fats are preferred for resistance to oxidation and for greater approximation to breast milk components.

When vegetable oils and fats used as sources of saturated fatty acids for transesterification are compared, such as saturated fatty acid-rich palm oil and relatively unsaturated fatty acid-rich soybean oil, the rate of reduction of AAA during transesterification is roughly the same for both oils, with no difference in the AAA content seen, and therefore it is believed that these exhibit an improving effect toward a more highly digestible and absorbable oil/fat. However, some difference is found in the resistance against oxidative damage due to differences in the fatty acids introduced by the transesterification reaction.

MCT, in particular triglycerides containing octanoic acid as a constituent fatty acid, and microbial arachidonic acid triglycerides are transesterified by a lipase according to the present invention to produce triglycerides containing arachidonic acid as a constituent fatty acid, such as "8A8", "88A". This method is useful for producing oils and fats containing

arachidonic acid economically without waste of arachidonic acid, and arachidonic acid-containing oils and fats resulting therefrom are useful as novel oils and fats having improved digestibility and absorbability.

5 There may also be mentioned oils and fats separated from these oils and fats or having increased saturated fatty acid contents by partial hydrogenation, although the usable oils and fats are not limited to these.

<Oils/fats comprising polyunsaturated fatty acids as  
10 constituent fatty acids>

It is essential to culture microbes capable of producing the oils/fats or triglyceride containing the polyunsaturated fatty acids as a constituent fatty acid. Here, the microbes are preferably microbes that produce  
15 at least one type of  $\omega 6$ ,  $\omega 9$  or  $\omega 3$  series polyunsaturated fatty acids having 20 or more carbons and 2 or more double bonds, primarily as a triglyceride constituent fatty acid.

As  $\omega 6$ ,  $\omega 9$  or  $\omega 3$  series polyunsaturated fatty acids  
20 having 20 or more carbons and 2 or more double bonds there may be mentioned dihomo- $\gamma$ -linolenic acid (8,11,14-eicosatrienoic acid:DGLA), arachidonic acid (5,8,11,14-eicosatetraenoic acid), 7,10,13,16-docosatetraenoic acid (22:4  $\omega 6$ ), DPA  $\omega 6$  (4,7,10,13,16-docosapentaenoic acid),  
25 8,11-eicosadienoic acid, mead acid (5,8,11-eicosatrienoic acid), 8,11,14,17-eicosatetraenoic acid (20:4  $\omega 3$ ), EPA (5,8,11,14,17-eicosapentaenoic acid), DPA  $\omega 3$  (7,10,13,16,19-docosapentaenoic acid) and DHA (4,7,10,13,16,19-docosahexaenoic acid).

30 According to the present invention, therefore, any microbe may be used which can produce an oil/fat or triglyceride containing polyunsaturated fatty acids as a constituent fatty acid. As examples of microbes capable of producing oils/fats or triglycerides containing  
35 arachidonic acid as a constituent fatty acid there may be

mentioned microbes belonging to the genus *Mortierella*,  
*Conidiobolus*, *Pythium*, *Phytophthora*, *Penicillium*,  
*Cladosporium*, *Mucor*, *Fusarium*, *Aspergillus*, *Rhodotorula*,  
*Entomophthora*, *Echinosporangium* or *Saprolegnia*. As  
5 microbes belonging to the genus *Mortierella* subgenus  
*Mortierella* there may be mentioned *Mortierella elongata*,  
*Mortierella exigua*, *Mortierella hygrophila*, *Mortierella*  
*alpina*, and the like. Specifically, there may be  
mentioned strains such as *Mortierella elongata* IFO8570,  
10 *Mortierella exigua* IFO8571, *Mortierella hygrophila*  
IFO5941, *Mortierella alpina* IFO8568, ATCC16266,  
ATCC32221, ATCC42430, CBS219.35, CBS224.37, CBS250.53,  
CBS343.66, CBS527.72, CBS529.72, CBS608.70, CBS754.68,  
etc. As examples of DHA-producing microbes there may be  
15 mentioned those belonging to the genres *Crypthecodenium*,  
*Thrautochytrium*, *Schizochytrium*, *Ulkenia*, *Japonochytrium*,  
*Haliphthoros*, and the like.

These strains are all available without restriction  
from the Institute for Fermentation, Osaka (IFO), the  
20 American Type Culture Collection (ATCC) and the  
Centraalbureau voor Schimmelcultures (CBS). In addition,  
there may be used strain *Mortierella elongata* SAM0219  
(FERM P-8703) (FERM BP-1239) separated from soil by the  
research group of the present inventors.

25 For culturing of the strains used for the invention,  
the spores, hypha or seed culture solution obtained by  
preculturing or cells collected from the seed culture  
solution are inocultured to liquid or solid medium for  
main culturing. In the case of a liquid medium, the  
30 carbon source used may be any commonly used one such as  
glucose, fructose, xylose, saccharose, maltose,  
solubilized starch, molasses, glycerol, mannitol or  
saccharized starch, although there is no limitation to  
these. As nitrogen sources there may be used organic  
35 nitrogen sources, including natural nitrogen sources such  
as peptone, yeast extract, malt extract, meat extract,  
casamino acid, corn steep liquor, soybean protein,

defatted soybean, cottonseed meal or the like, as well as urea, and inorganic nitrogen sources such as sodium nitrate, ammonium nitrate and ammonium sulfate, and in particular there may be mentioned soybean-derived  
5 nitrogen sources such as soybean, defatted soybean, soybean flakes, edible soybean protein, okara (bean-curd refuse), soybean milk, parched soybean flour and the like. More particularly there may be used one or more types of heat denatured defatted soybean, and more  
10 preferably defatted soybean heat treated at about 70-90°C and removed of its ethanol-soluble components, or combinations with the aforementioned nitrogen sources. If necessary, phosphorus ion, potassium ion, sodium ion, magnesium ion and calcium ion, as well as ions of metals  
15 such as iron, copper, zinc, manganese, nickel, cobalt and the like or vitamins may also be used as trace nutrient sources.

These medium components are not particularly restricted so long as they are at concentrations which do  
20 not inhibit growth of the microbes. In practice, the carbon source will generally be added in a total amount of 0.1-40 wt% and preferably 1-25 wt%, and the nitrogen source in a total amount of 2-15 wt% and preferably 2-10 wt% and, more preferably, the initial carbon source is  
25 added at 1-5 wt% and the initial nitrogen source at 3-8 wt%, after which the carbon and nitrogen source and more preferably only the carbon source is added during the culturing. In order to increase the polyunsaturated fatty acid yield, for example, a hydrocarbon such as  
30 hexadecane or octadecane; a fatty acid such as oleic acid or linoleic acid or a salt thereof, or a fatty acid ester such as an ethyl ester, glycerin fatty acid ester or sorbitan fatty acid ester; or an oil such as olive oil, soybean oil, rapeseed oil, cottonseed oil or coconut oil,  
35 may be used as an unsaturated fatty acid precursor, either alone or in combinations. The amount of substrate added may be 0.001-10 wt% and preferably 0.5-10 wt% with

respect to the medium. Any of these substrates may also be used as the sole carbon source for culturing.

The culturing temperature for the polyunsaturated fatty acids-producing microbe will differ depending on the microbe, but may be 5-40°C and preferably 20-30°C, or the cells may be cultured at 20-30°C for growth and the culturing continued at 5-20°C to produce the unsaturated fatty acid. Such temperature management can also be used to increase the proportion of polyunsaturated fatty acids among the produced fatty acids. The pH of the medium may be 4-10 and preferably 5-9, and the culturing method may be submerged culturing, shake culturing, solid culturing or stationary liquid culturing. The culturing will usually be conducted for 2-30 days, preferably for 5-20 days and more preferably for 5-15 days.

Microbes belonging to the genus *Mortierella* subgenus *Mortierella* are known as microbes capable of producing oils/fats or triglycerides containing arachidonic acid as the main constituent fatty acid but, by mutating the aforementioned strains, the present inventors have obtained microbes capable of producing oils/fats or triglycerides comprising dihomog- $\gamma$ -linolenic acid as the main constituent fatty acid (see Japanese Unexamined Patent Publication No. 5-91887) and capable of producing oils/fats or triglycerides comprising  $\omega$ 9 series polyunsaturated fatty acids as the main constituent fatty acids (see Japanese Unexamined Patent Publication No. 5-91888). In addition, the inventors have obtained microbes with resistance to high-concentration carbon sources (PCT Patent Publication WO 98/39468) which are microbes belonging to the genus *Mortierella* subgenus *Mortierella*. The present invention, however, is not limited to microbes of the genus *Mortierella* subgenus *Mortierella*, and any microbes capable of producing oils/fats or triglycerides containing polyunsaturated fatty acids as the constituent fatty acids may be used.

As the method of obtaining the crude oil from the microbe cultured in the manner described above, the cultured solution is subjected to ordinary solid/liquid separation means such as natural sedimentation, centrifugal separation and/or filtration, either directly or after a treatment such as sterilization, concentration, acidification or the like, to obtain the cultured cells. A flocculent or filtering aid may also be added to facilitate the solid/liquid separation. As flocculating agents there may be used, for example, aluminum chloride, calcium chloride, algin, chitosan and the like. As filtering aids there may be used, for example, diatomaceous earth. The cultured cells are preferably rinsed, crushed and dried. The drying may be carried out by lyophilization, blow drying, fluidized bed drying or the like. The means for obtaining the crude oil from the dried cells may be extraction with an organic solvent or compression, but preferably extraction is performed with an organic solvent under a nitrogen stream. As organic solvents there may be used ethanol, hexane, methanol, chloroform, dichloromethane, petroleum ether, acetone or the like, and methanol/petroleum ether alternating extraction or chloroform-methanol-water monolayer solvent may also be employed. However, the extraction method used to obtain the crude oil is not limited to these methods, and any method for efficient extraction of oils and fats from cells may be used. For example, supercritical extraction may be employed as an effective means.

The desired crude oil can be obtained by removing the organic solvent or supercritical fluid components from the extract obtained by extraction with an organic solvent or supercritical fluid, under reduced pressure conditions or the like. Extraction may also be performed using wet cells as an alternative to the methods mentioned above. In this case, there is used a water-compatible solvent such as methanol, ethanol or acetone,

or a water-compatible mixed solvent comprising such solvents with water and/or another solvent. The procedure is otherwise the same as described above.

A purified oil or fat instead of a crude oil may also be used as the substrate for transesterification. The oil or fat purification step may be accomplished by ordinary methods such as degumming, deacidification, steam distillation, decoloration, column treatment, molecular distillation, wintering or the like.

#### <Lipases used for transesterification>

As examples of lipases to be used for the invention there may be mentioned those produced by microbes belonging to the genus *Rhizopus*, *Rhizomucor*, *Aspergillus* or the like, as well as pig pancreatic lipases. Such lipases may be commercially available ones. Examples thereof include *Rhizopus delemar* lipase (Talipase, product of Tanabe Seiyaku) (the current taxonomic classification of *Rhizopus delemar* is *Rhizopus oryzae*), *Rhizopus niveus* lipase (Newlase F3G, product of Amano Enzyme Co., Ltd.) (*Rhizopus niveus* has the same taxonomical classification as *Rhizopus delemar*, both being designated as *Rhizopus oryzae*), *Rhizomucor miehei* lipase (Ribozyme IM by Novo Nordisk Co., Ltd., or Chirazyme L-9, c.-f., C2, lyo, by Berlinger Mannheim) and *Aspergillus niger* lipase (Lipase A, product of Amano Enzyme Co., Ltd.), with no particular limitation to these enzymes, as any 1,3-position specific type lipases may be used.

The form in which the lipase is used is preferably as an immobilized lipase on an immobilizing resin, in order to increase the reaction efficiency for the polyunsaturated fatty acids and to confer stability for repeated use. As immobilizing resins there may be used celite, ion-exchange resins, ceramics, protein-adsorbing adsorption resins and the like. As examples there may be mentioned powdered diatomaceous earth and granular diatomaceous earth as Celite, Dowex MARATHON WBA (Dow

Chemical) and DIAION WA30 (Mitsubishi Chemical) as ion-exchange resins, SM-10 (Nihon Gaishi) as a ceramic and DIAION HP20 (Mitsubishi Chemical) as an adsorption resin.

5 The aforementioned ion-exchange resins, etc. are only examples, and new improved resins are continually being developed and are commercially available. Such improved resins may also be considered suitable for use.  
<Reaction temperature and time for transesterification>

10 The transesterification is preferably conducted at 30-60°C in consideration of the enzyme reactivity. It is more preferably conducted at 30-50°C in consideration of stability of the immobilized enzyme when the reaction is carried out continuously using an immobilized enzyme.  
15 The time required for the reaction is preferably as short as possible, but the reaction is preferably conducted for 1-7 days in consideration of the XXA or AAA content of the transesterified oil/fat after the reaction. Because the raw material oil and fat (polyunsaturated fatty acids-rich oils and fats) are prone to oxidation during  
20 the reaction, oxygen is preferably removed in the manner described above and the reaction is preferably carried out under oxygen-free conditions.

<Purification after transesterification reaction>

25 The oils or fats improved by transesterification in this manner may be easily separated from the immobilized enzyme in the enzyme reactor (whether a column reaction or batch reaction). Most of the components of the prepared oils or fats will be in the form of triglycerides, and these may be purified by ordinary  
30 methods used for purification of oils and fats, such as deacidification, degumming, decoloration, steam distillation and the like.

35 The oils and fats of the invention are improved oils/fats or triglycerides obtained by lipase transesterification of oils/fats or triglycerides comprising microbe-produced polyunsaturated fatty acids as the constituent fatty acids, and they are oils/fats or

triglycerides containing at least 20% of polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds, and there may be mentioned transesterified oils/fats or triglycerides containing at least 40% of triglycerides with one residue of polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds in the molecule, and/or no more than 4.0% of triglycerides with 3 residues of the same polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds.

The oils and fats of the invention preferably comprise at least 90% triglycerides. The oils and fats may be any form thereof, but in the present invention, they comprise diglycerides, monoglycerides, and possibly a trace amount of free fatty acids, as resultant products by transesterification, as well as the triglycerides as a major product thereof.

The oils and fats of the invention are, for example, oils/fats or triglycerides containing at least 20% of arachidonic acid, and transesterified oils/fats or triglycerides containing at least 40% of triglycerides with one residue of arachidonic acid in the molecule and/or no more than 4.0% of AAA have unlimited possibilities for use as raw materials and additives in foods, feeds, cosmetics and pharmaceuticals. Furthermore, there are no limitations on the purpose or amounts of their use.

As examples of food compositions there may be mentioned common food products as well as functional foods, nutritional supplements, modified milk for immature infants, modified milk for infants, infant food products, maternal foods or geriatric foods. As examples of food products containing oils or fats there may be mentioned natural foods which naturally contain oils and fats, such as meat, fish and nuts, food products with oils and fats added during preparation, such as soups, food products using oils or fats as heating media, such

as donuts, oil or fat food products such as butter, processed food products with oils or fats added during processing, such as cookies, or food products sprayed or coated with oils or fats during final processing, such as  
5 hard biscuits. They may also be added to agricultural foods, fermented foods, livestock foods, marine foods or beverages which contain no oils or fats. They may also be in the form of functional food products or pharmaceuticals, and for example, in the form of enteral  
10 nutrients, powders, granules, lozenges, oral solutions, suspensions, emulsions, syrups or the like.

The present invention will now be explained in greater detail through the following examples, with the understanding that the invention is in no way restricted  
15 by the examples.

#### EXAMPLES

Example 1 Preparation of immobilized enzyme (Conferred stability and deoxygenation by immobilization of 1,3-  
20 specific lipase)

After suspending 100 g of an ion-exchange resin (Dowex MARATHON WBA: Dow Chemical) in 80 ml of *Rhizopus delemar* lipase solution (12.5% Talipase powder, product of Tanabe Seiyaku), the suspension was dried under  
25 reduced pressure to obtain the immobilized enzyme. Next, a triglyceride containing 40% arachidonic acid (SUNTGA40S, trade name of Suntory Co., Ltd.) (25 g), palm oil (15 g), the aforementioned immobilized lipase (4 g) and water (800  $\mu$ l) were weighed out into a sealable  
30 bottle. The half-open bottle was placed in a dessicator, and a vacuum pump was connected to the dessicator for pressure reduction of the contents. (Bubbles were produced in the oil from the immobilized enzyme resin placed in the bottle inside the desiccator together with  
35 the oil, thus releasing the air containing the immobilized enzyme.) Nitrogen gas was then leaked into the desiccator to restore the interior of the desiccator

to ordinary pressure. This procedure of pressure reduction and restoration was repeated for substitution of nitrogen for the oxygen present in the immobilized resin matrix, and the bottle was sealed. For activation of the immobilized enzyme, the bottle was then shaken (100 rpm) at 30°C for 24 hours. After completion of activation, the oil used for activation was removed and the deoxygenation procedure was carried out once more to obtain the activated immobilized enzyme. For transesterification with other vegetable oil or fat, the vegetable fat or oil used for transesterification was used instead of palm oil for activation of the immobilized enzyme, in a mixture of the SUNTGA40S (25 g), vegetable oil (15 g), immobilized lipase (2 g) and water (800 µl) as with the palm oil mentioned above, and the aforementioned deoxygenation and immobilized enzyme activation procedures were carried out for transesterification.

## Example 2 Method of measuring fatty acid contents of oils/fats

The fat or oil was sampled in an approximately 10-20 mg screw cap test tube, 2 mL of methanolic HCl and 1 mL of dichloromethane were added, the top of the test tube was sealed, and reaction was conducted at 50°C for 3 hours. After completion of the reaction, the mixture was cooled to room temperature and then hexane was added for extraction of the fatty acid methyl ester and the collected hexane layer was concentrated under reduced pressure. The obtained fatty acid methyl ester was dissolved in hexane to a total fatty acid methyl ester concentration of about 1% and provided for gas chromatography (GC) analysis. The GC analysis conditions and component identification were according to "Fatty Acid Composition by GLC (Method Ce 1b-89)" of Official Methods and Recommended Practices of the AOCS, 5th Edition (American Oil Chemists' Society, 1998), a

Supelcowax-10 column was used. The ratio of the arachidonic acid methyl ester peak area with respect to the total peak area detected was recorded as the arachidonic acid content in the oil/fat. The fatty acid contents of fatty acids other than arachidonic acid were determined by the same method.

### Example 3 Method of triglyceride analysis

HPLC analysis was conducted under the following conditions to measure the content of each triglyceride in the oils or fats modified by the enzyme reaction.

Column: Reverse-phase column (Cosmosil 4.6 x 250 mm 5C18-MS)

Solvent: Acetone:acetonitrile (1:1) 1 ml/min

Analysis time: 55 min

Column oven temperature: 40°C

Detector: Refractive index detector (cell temperature: 40°C)

Sample: 5 µl of 10% solution of oil/fat or triglyceride in chloroform

Each triglyceride was analyzed under the above-mentioned measuring conditions. The molecular species of each triglyceride was determined according to the method disclosed in Japanese Unexamined Patent Publication No. 2000-513575. Specifically, the peaks of each triglyceride were separated, and hydrolysis was then followed by methyl ester conversion. The molecular species were determined by GC.

By analyzing the triglycerides of the oils/fats obtained with SUNTGA40S and transesterification by the method described above and performing calculation, it was possible to determine the form of the SUNTGA40S arachidonic acid and the proportion and content of each XXA triglyceride. XXA represents the total weight percentage of LLA, PGA, OLA, PLA, OOA, POA, PPA, SOA, PSA, SSA and LC22A. The notations used here are as

follows. L: linoleic acid, P: palmitic acid, G:  $\gamma$ -linolenic acid, O: oleic acid, S: stearic acid, C22: linear saturated C<sub>22</sub> fatty acid, X: fatty acid other than arachidonic acid, A: arachidonic acid. (The binding positions of each fatty acid residue on the glycerin were not stipulated.)

Example 4 Modification of arachidonic acid-containing oil/fat (SUNTGA40S; approximately 40% as arachidonic acid) using activated immobilized lipase obtained in Example 1

Enzyme reaction was conducted by shaking (100 rpm) at 30°C with the raw material composition shown in Table 3 below, and the reaction was continued while sampling to confirm the extent of the transesterification reaction. The AAA conversion was measured by analysis of triglycerides of the sampled oil.

Table 3 Raw material composition for reaction

SUNTGA40S	25 g
Palm oil or soybean oil	15 g
Immobilized lipase	2 g

Fig. 1 shows conversion of the AAA content upon combining and transesterifying vegetable oil such as palm oil or soybean oil with arachidonic acid-containing oil as a type of polyunsaturated fatty acids-rich oil/fat or triglyceride. Even transesterification with the saturated fatty acid-rich palm oil resulted in the AAA reduction rate with the unsaturated fatty acid-rich linoleic acid-based soybean oil.

This demonstrated that the rate of AAA disappearance according to the invention is identical regardless of the type of vegetable oil used, so long as the mixing proportion is the same.

Example 5 CDM test

In order to confirm the oxidative stability of the enzyme-converted arachidonic acid-rich oil, a test sample was prepared and the oxidative stability was measured by a CDM test. The CDM test was conducted by heating the oil sample in a reactor (at constant temperature) while blowing in clean air and capturing in water the volatile decomposition products produced by oxidation. The stability of the oil sample was expressed in terms of the time lapse up to the curve point at which the conductivity of the water changed drastically. A shorter time indicates an oil more susceptible to oxidative damage.

Based on Example 3, a 2-fold amount of the immobilized enzyme (activated) was added (4 g) to the raw material oil (40 g), and reaction was conducted at 30°C, 100 rpm. After the reaction, the oil and immobilized enzyme were separated with a decant to obtain the reaction oil. The AAA content of the obtained oil was analyzed by HPLC and the stability was measured by a CDM test. The reaction time was varied to obtain modified oil 1 and modified oil 2 having different AAA contents.

Table 4 AAA contents and stabilities of modified oils (transesterified oils)

Sample	CDM (hr)	AAA content (%)
<u>Palm oil/SUNTGA40S</u>		
Modified oil 1	6.4	3.90
Modified oil 2	5.7	4.44
Unmodified oil	4.6	6.46
<u>Soybean oil/SUNTGA40S</u>		
Modified oil 1	4.1	3.69
Modified oil 2	3.6	4.28
Unmodified oil	3.5	7.01

As shown in Table 4, when the saturated fatty acid oil, palm oil, was used as the raw material vegetable oil for reaction, the problematic AAA content was reduced and the stability of the oil against oxidation was increased.

On the other hand, when the linoleic acid oil,

soybean oil, was used as the raw material for reaction, a similar reduction in AAA was seen as with palm oil, but the effect on stability as measured by CDM was not as notable as with palm oil.

5

Example 6 Changes in components with time for transesterification of arachidonic acid-rich oil (SUNTGA40S) diluted with palm oil

The fungus-produced arachidonic acid-rich oil contained abundant AAA, and therefore the AAA was converted to XXA by lipase transesterification reaction in order to approach the form of arachidonic acid (XXA) in breast milk, and the progression and changes in component proportions with time were experimentally measured.

15

Table 5 Changes in triglyceride components with time during transesterification reaction

Triglyceride component (%)	Time (hr)			
	0 hr	48 hr	96 hr	176 hr
AAA	6.37	5.16	4.33	3.41
XAA	23.19	19.09	17.00	14.86
XXA	16.11	29.33	35.41	40.55

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The immobilized enzyme was activated by the method in Example 1, and then 25 g of SUNTGA40S and 15 g of palm oil were added to 2 g of the activated immobilized enzyme and deoxygenation was followed by reaction at 30°C.

25

Sampling was conducted at various times, the triglycerides were analyzed by the method described in Example 2 (HPLC analysis), and each of the component contents were calculated. As shown in Table 5, AAA decreased with time while the presumed conversion product XXA increased. Under these conditions, a period of 1 week was required for approximately 1/2 reduction of AAA and at least doubling of XXA, from mixing of the oils. The following example was then carried out to determine

30

optimization of the reaction temperature and amount of immobilized enzyme addition.

Example 7 Effect of reaction temperature during transesterification reaction

The immobilized enzyme prepared in Example 1 was used to examine the effect of the reaction temperature on transesterification. The immobilized enzyme was activated by the method in Example 1, and then 25 g of SUNTGA40S and 15 g of palm oil were added to 2 g of the activated immobilized enzyme and deoxygenation was followed by reaction at different temperatures. The reacted oil was sampled after 1 day of reaction, the triglycerides were analyzed by the method described in Example 2 (HPLC analysis), and the AAA residue was calculated (Table 6).

Table 6 Effect of temperature on esterification reaction

Reaction temperature	AAA residue (%)	AAA reduction (% , per 24 hrs)
Before reaction	6.37	
30°C	5.86	9%
45°C	4.50	29%
55°C	2.43	62%

Since a transesterification reaction is ordinarily conducted at below 60°C, the reaction was conducted at 30°C, 45°C and 55°C and reactivity was confirmed. As shown in the table, the transesterification reaction was confirmed to proceed more efficiently with a higher reaction temperature. The deoxygenation step is therefore important from the standpoint of preventing oxidative damage during reaction at high temperature.

Example 8 Examination with enzyme agent comprising immobilized *Rhizomucor miehei* lipase

In the same manner as Example 5, with the enzyme added at 10% and using SUNTGA40S (trade name of Suntory Co., Ltd.) diluted with palm oil, a comparison was made

of the transesterification activity of *R. delemar* lipase immobilized and activated according to Example 1, and a commercially available *R. miehei* immobilized lipase (Chirazyme L-9, c.-f., C2, lyo, product of Berlinger Mannheim) which requires no activation with water.

Table 7 Comparison of transesterification reactions with *R. delemar* and *R. miehei*

Molecular species	<i>R. delemar</i>	<i>R. miehei</i>
AAA	2.86	2.53
XAA	14.95	14.05
XXA	43.33	44.20

Each triglyceride was analyzed by the method described in Example 3 (HPLC analysis), and each component content was calculated. As shown in Table 7, both enzyme reactions were conducted for 48 hours at 45°C, and the rates of AAA decrease and XXA increase were approximate equivalent for both enzymes, indicating their suitability for transesterification reaction.

#### Example 9 Improvement of arachidonic acid by transesterification reaction

The present inventors have developed a method for obtaining microbes capable of producing oils/fats or triglycerides containing arachidonic acid as the major constituent fatty acid and for obtaining purified oils and fats by fermentative production on an industrial scale, by culturing microbes belonging to the genus *Mortierella* (see Higashiyama et al., Enhancement of Arachidonic Acid Production by *Mortierella alpina* 1S-4, J. Am. Oil Chem. Soc., 75, pp.1501-1505(1998)). Using a oil/fat or triglyceride containing arachidonic acid as the main constituent fatty acid obtained by the aforementioned method as the raw material oil/fat and a deoxygenated, activated immobilized enzyme (*R. delemar* lipase) obtained by the method of Example 1,

transesterification was accomplished with palm oil as a vegetable oil/fat. The oil/fat containing 40 wt% arachidonic acid as the main constituent fatty acid (250 g), palm oil (150 g) and the immobilized enzyme (40 g) were reacted at 45°C for 48 hours. A 97% portion of the raw material oil/fat of the reaction was in triglyceride form, with arachidonic acid present as the main constituent fatty acid and tri-arachidonic acid (AAA) constituting 6.37% of the triglycerides. The oil obtained by transesterification was filtered to separate the immobilized enzyme, yielding 385 g of arachidonic acid-rich oil/fat or triglyceride containing 95.1% triglyceride, 2.83% AAA and 42.3% XXA (monoarachidonic acid having a single bonded arachidonic acid residue).

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Example 10 Improvement of dihomog- $\gamma$ -linolenic acid (DGLA)-rich oils/fats by transesterification

The present inventors have also developed a method for obtaining microbes capable of producing oils/fats or triglycerides containing dihomog- $\gamma$ -linolenic acid (DGLA) as the major constituent fatty acid and for obtaining purified oils and fats by fermentative production on an industrial scale, by mutating microbes belonging to the genus *Mortierella* subgenus *Mortierella* (see Japanese Unexamined Patent Publication No. 5-91887). Using a oil/fat or triglyceride comprising DGLA as the main constituent fatty acid obtained by the aforementioned method as the raw material oil/fat and a deoxygenated, activated immobilized enzyme (*R. delemar* lipase) obtained by the method of Example 1, transesterification was accomplished with palm oil as a vegetable oil/fat. The oil/fat comprising 40 wt% DGLA as the main constituent fatty acid (25 g), palm oil (15 g) and the immobilized enzyme (4 g) were reacted at 45°C for 48 hours. At the start of the reaction, triglycerides constituted 97.2%, DGLA constituted 25% of the constituent fatty acids, and

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tri-dihomo- $\gamma$ -linolenic acid (DDD) constituted 6.09% of the triglycerides. Upon reaction, triglycerides constituted 95.3% and DDD was reduced to 3.03% of the triglycerides, and therefore the DGLA in the oil/fat comprising DGLA as the main constituent fatty acid was reduced, similar to the arachidonic acid-containing oil/fat or triglycerides.

Example 11 Preparation of arachidonic acid-containing oil/fat (crude oil)

*Mortierella alpina* CBS754.68 was used as the arachidonic acid-producing strain. The preserved cells were inoculated to a 1% yeast extract, 2% glucose medium at pH 6.3, and seed culturing (first stage) was initiated at a stirring speed of 100 rpm and a temperature of 28°C and continued for 3 days. After preparing 30 L of a 1% yeast extract, 2% glucose, 0.1% soybean oil medium at pH 6.3 in a 50 L volume submerged culturing vat, the seed culture solution (first stage) was added thereto and seed culturing (second stage) was initiated at a stirring speed of 200 rpm, a temperature of 28°C and an internal pressure of 150 kPa, and continued for 2 days. Next, 4500 L of medium (Medium A: 270 kg soybean flour, 16.2 kg  $\text{KH}_2\text{PO}_4$ , 2.7 kg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 2.7 kg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 54 kg soybean oil) was sterilized at 121°C for 20 minutes. Also, 800 L of a separate medium (Medium B: 108 kg of hydrous glucose) was sterilized at 140°C for 40 seconds and added to the previous Medium A to prepare Medium C. After adjusting Medium C to pH 6.1, the seed culture solution (second stage) was transferred thereto to a total of 5400 L of initial culturing volume (10 kL culturing vat volume). Culturing was initiated at a temperature of 26°C, an airflow of 49  $\text{Nm}^3/\text{hr}$  and an internal pressure of 200 kPa. Feeding culture was performed as shown Table 8, and the main culturing was conducted for 210 hours. After completion of the culturing, the culture solution volume was 7100 L as a result of the increase due to

feeding culture and the reduction due to evaporation.

Table 8 Feeding culture of Example 1

Main culturing time	Feeding culture
After 19 hours	(243 kg hydrous glucose + 54 kg soybean oil)/400 L
After 43 hours	243 kg hydrous glucose/400 L
After 67 hours	216 kg hydrous glucose/380 L
After 91 hours	162 kg hydrous glucose/300 L
After 120 hours	108 kg hydrous glucose/200 L

5           After completion of the culturing, sterilization was carried out at 120°C for 20 minutes and then the wet cells were collected with a filter press and dried to a moisture content of 2 wt% using a fluidized bed drier, and a pneumatic conveyor was used to convey the dry cells  
10 to the packing location. The obtained dry cells were packed into an approximately 100 L volume aluminum pouch together with nitrogen gas, and after sealing the mouth of the bag, it was stored in a refrigerator at below 10°C.

15           The dry cells taken out of the container bag were subjected to extraction with hexane, and after filtering the hexane solution to remove the solid portion, it was heated under reduced pressure to remove the hexane and obtain a crude oil containing arachidonic acid as a  
20 constituent fatty acid.

          Analysis of the crude oil revealed 94% triglycerides and a 29.6% arachidonic acid content of the total fatty acids.

25   Example 12   Transesterification of oil prepared in Example 11

          The crude oil containing 29.6% arachidonic acid prepared in Example 11 (94% triglycerides) was subjected to lipase transesterification without addition of  
30 vegetable oil or fat, to attempt improvement of the crude oil. In immobilized lipase of *R. delemar* was used for deoxygenation and activation by the method described in

Example 1, and the immobilized enzyme (4 g) was added to the crude oil containing 29.6% arachidonic acid (40 g) and reaction was conducted with shaking at 45°C for 48 hours. The oil with a composition of 6.08% AAA and 24.15% XXA at the start of the reaction was converted to a composition of 3.21% AAA and 40.37% XXA by completion of the reaction. When the crude oil was used, it was found that the improved oil had been transesterified similar to the transesterification of oils comprising microbe-produced polyunsaturated fatty acids as the main constituent fatty acids as in Examples 6 and 8, without specific addition of vegetable oil or fat.

#### Example 13 Addition to milk

A 0.44 g portion of modified oil 1 (novel transesterified oil) obtained in Example 9 was added to 100 g of powdered milk to prepare a modified milk approximating human breast milk. The modified milk comprised arachidonic acid in a proportion of 0.5% with respect to the total fatty acids.

#### Example 14 Addition to butter

A 1.97 g portion of modified oil 1 (novel transesterified oil) obtained in Example 9 was added to 100 g of butter fat from which the butter milk had been removed by churning during the butter production process, and the mixture was worked to a homogeneous composition to obtain arachidonic acid-added butter.

#### Example 15 Addition to juice

A 2 g portion of  $\beta$ -dextrin was added to 20 ml of a 20% aqueous ethanol solution, and then 100 mg of modified oil 1 (novel transesterified oil) obtained in Example 9, containing 0.05 wt% vitamin E, was added thereto while stirring with a stirrer, and the mixture was incubated at 50°C for 2 hours. After cooling to room temperature (approximately 1 hour), incubation was continued at 4°C

for 10 hours while continuing to stir. The produced precipitate was recovered by centrifugal separation, and after washing with n-hexane, it was lyophilized to obtain 1.8 g of a cyclodextrin clathrated compound comprising arachidonic acid-containing triglycerides. A 1 g portion of this powder was uniformly mixed with 10 L of juice to prepare juice comprising arachidonic acid-containing transesterified oil.

Example 16 Preparation of triglycerides containing medium-chain fatty acids and arachidonic acid in the same molecule by transesterification, and purification of the oil/fat

The immobilized lipase prepared by the method of Example 1 was subjected to deoxygenation and activation according to Example 1. Specifically, triglyceride contains 40% arachidonic acid (SUNTGA40S, trade name of Suntory Co., Ltd.) (200 g), MCT (COCONARD RT, trade name of Kao Corp.) (200 g), the aforementioned immobilized lipase (40 g) and water (8 ml) were placed in a sealable bottle and subjected to the deoxygenation and enzyme activation procedure described in Example 1.

The oil used for the activation procedure was removed, and then SUNTGA40S (200 g) and MCT (200 g) were freshly added to the immobilized enzyme-containing bottle prior to shaking at 30°C for 48 hours (100 rpm).

After completion of the reaction, the oil was taken out from the bottle and the triglycerides of the transesterified oil were analyzed by the HPLC described in Example 3. The analysis results are shown in Table 9. The HPLC method does not allow separate analysis of triglycerides containing medium-chain fatty acids and arachidonic acid (for example, triglycerides with octanoic acid at positions 1,2 and arachidonic acid at position 3 (88A), triglycerides with octanoic acid at positions 1,3 and arachidonic acid at position 2 (8A8), etc.) obtained by transesterification. This was

therefore combined with GC (conditions shown below) analysis for separate analysis of 88A and 8A8. 88A appeared on the chromatograph at 17.01 minutes, and 8A8 at 17.15 minutes.

5           The content of each triglyceride resulting from the reaction was as shown in Table 10.

[GC analysis method]

          Column: Frontier Ultra ALLOY UA-17-15M-0.1F (15 m x 0.25 mm x 0.1  $\mu$ m)

10           Column temperature: 260°C-(1°C/min)-290°C-(10°C/min)-390°C (5' min)

          Analysis time: 45 minutes

          Injection port temperature: 310°C

15           Detector temperature: 370°C (Hydrogen ionization detector)

          Carrier gas: helium

          Line speed: 40 cm/min

          Sample: Injection of 1  $\mu$ l of 1% solution of oil (triglyceride) dissolved in hexane

20

          The transesterified oil remaining after analysis was subjected to molecular distillation to remove approximately 85% of the MCTs remaining after the reaction.

Table 9 Proportions of triglycerides  
Results of HPLC analysis

Triglyceride	Before reaction (wt%)	After reaction (wt%)
8A8, etc.	0.00	15.87
8P8	0.00	6.96
8L8	0.00	6.58
8O8	0.00	15.23
AAA	4.84	1.67
8AA	0.00	6.25
GAA	0.79	0.00
LAA	3.06	2.86
PAA	5.55	4.71
SAA	3.97	1.45
C22AA	1.30	0.28
C24AA	3.72	0.52
LLA	0.55	1.45
PGA	1.88	0.91
OLA	0.67	0.28
PLA	3.04	1.01
OOA	0.60	0.00
POA	2.97	0.84
PPA	1.32	0.41
SOA	0.90	0.70
PSA	1.30	0.23
SSA	0.93	0.44
LAC22	0.57	0.27
888	50.00	23.27
Other triglycerides	12.04	7.81

8A8, etc.: Mixture of 8A8 (88A, 8A8), 8G8, 8D8

5 The fatty acids were indicated by alphanumeric notation (without specifying the binding position on the glycerin backbone).

(The glycerin backbone positions of the triglycerides other than 88A and 8A8 in the table are not specified in the table.)

Fatty acid notations:

10 8: octanoic acid, A: arachidonic acid, D: DGLA, P: palmitic acid, S: stearic acid, O: oleic acid, G:  $\gamma$ -linolenic acid, L: linoleic acid, C22: C<sub>22</sub> saturated linear fatty acid, C24: C<sub>24</sub> saturated linear fatty acid

Table 10 Proportions of triglycerides  
Results of GC and HPLC analysis

Triglyceride	Before reaction (wt%)	After reaction (wt%)
88A	0.00	8.36
8A8	0.00	5.37
88G + 8G8	0.00	0.35
88D + 8D8	0.00	1.79
88L + 8L8	0.00	6.58
88P + 8P8	0.00	6.96
88O + 8O8	0.00	15.23
8AA + A8A	0.00	6.25
AAA	4.84	1.67
XAA + AXA	18.39	9.82
XXA + XAX	14.73	6.54
888	50.00	23.27
Other triglycerides	12.04	7.81

The triglycerides are represented by their constituent fatty acid types, with the binding positions of each fatty acid shown in this table in the order 1,2,3 or 3,2,1. The 2-position of glycerin in a triglyceride is an asymmetric carbon, and therefore the 1- and 3-positions are sterically different in the strict sense. However, as the 1- and 3-positions are basically equivalent in physiological terms as evidenced by recognition by lipases, triglycerides are considered the same even if both end positions are switched around the center fatty acid (position 2).

Explanation of notations in table

Fatty acids represented alphanumerically.

Fatty acid notations:

8: octanoic acid, A: arachidonic acid, D: DGLA, P: palmitic acid, S: stearic acid, O: oleic acid, G:  $\gamma$ -linolenic acid, L: linoleic acid, C22: C<sub>22</sub> saturated linear fatty acid, C24: C<sub>24</sub> saturated linear fatty acid

XAA + AXA: Total of GAA, LAA, PAA, SAA, C22AA, C24AA

XXA + XAX: Total of LLA, PGA, OLA, PLA, OOA, POA, PPA, SOA, PSA, SSA, LAC22

The fatty acid binding positions of specific triglycerides (for example, PGA) represented by XAA or XXA are not specified. The specific contents are shown in Table 9.

CLAIMS

1. A production process for a transesterified oil/fat or triglyceride, by transesterification of 50-100 parts by weight of one or more fungus-produced oils/fats or triglycerides containing at least 20% of polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds and 0-50 parts by weight of one or more vegetable oils/fats or triglycerides, using a 1,3-position specific type lipase.
2. A production process according to claim 1, wherein the transesterification reaction is conducted in a deoxygenated state.
3. A production process according to claim 1 or 2, wherein the fungus-produced fatty acid is  $\omega$ 6 series polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds.
4. A production process according to claim 1 or 2, wherein the fungus-produced fatty acid is  $\omega$ 9 series polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds.
5. A production process according to claim 1 or 2, wherein the fungus-produced fatty acid is arachidonic acid.
6. A production process according to claim 1 or 2, wherein the fatty acid is arachidonic acid produced by a microbe belonging to the genus *Mortierella*.
7. A production process according to claim 1 or 2, wherein the fungus-produced fatty acid is dihomog- $\gamma$ -linolenic acid.
8. A production process according to claim 1 or 2, wherein the fungus-produced fatty acid is mead acid.
9. A production process according to any one of claims 1 to 8, wherein the lipase is a lipase produced by *Rhizopus delemar*, *Rhizopus niveus*, *Rhizomucor miehei* or *Rhizopus oryzae*.
10. A transesterified oil/fat or triglyceride

obtained by a production process according to any one of claims 1 to 9.

5. 11. A transesterified oil/fat or triglyceride which is an oil/fat or triglyceride containing at least 20% of polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds, obtained by a production process according to any one of claims 1 to 9, and which contains at least 40% of triglycerides with one residue of polyunsaturated fatty acids containing 20 or more  
10 carbons and two or more double bonds in the molecule, and/or no more than 4.0% of triglycerides with 3 residues of the same polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds.

15 12. A transesterified oil/fat or triglyceride which contains at least 20% of fungus-produced  $\omega 6$  series polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds, obtained by a production process according to any one of claims 1 to 9, and which contains at least 40% of triglycerides with one residue  
20 of  $\omega 6$  series polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds in the molecule, and/or no more than 4.0% of triglycerides with 3 residues of the same fungus-produced  $\omega 6$  series polyunsaturated fatty acids containing 20 or more carbons  
25 and two or more double bonds.

30 13. A transesterified oil/fat or triglyceride which contains at least 15% of fungus-produced  $\omega 9$  series polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds, obtained by a production process according to any one of claims 1 to 9, and which contains at least 40% of triglycerides with one residue  
of  $\omega 9$  series polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds in the molecule, and/or no more than 4.0% of triglycerides with  
35 3 residues of the same fungus-produced  $\omega 9$  series

polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds.

5 14. A transesterified oil/fat or triglyceride containing at least 20% of arachidonic acid, obtained by a production process according to any one of claims 1 to 9, and which contains at least 40% of triglycerides with one residue of arachidonic acid in the molecule and/or no more than 4.0% of AAA (a triglyceride with 3 residues of arachidonic acid in the molecule).

10 15. A transesterified oil/fat or triglyceride containing at least 20% of dihomo- $\gamma$ -linolenic acid, obtained by a production process according to any one of claims 1 to 9, and which contains at least 40% of triglycerides with one residue of dihomo- $\gamma$ -linolenic acid  
15 in the molecule and/or no more than 4.0% of DDD (a triglyceride with 3 residues of dihomo- $\gamma$ -linolenic acid in the molecule).

20 16. A transesterified oil/fat or triglyceride containing at least 20% of mead acid, obtained by a production process according to any one of claims 1 to 9, and which contains at least 40% of triglycerides with one residue of mead acid in the molecule and/or no more than 4.0% of MMM (a triglyceride with 3 residues of mead acid in the molecule).

25 17. A transesterified oil/fat or triglyceride containing at least 20% of polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds, and which contains at least 40% of triglycerides with one residue of polyunsaturated fatty acids  
30 containing 20 or more carbons and two or more double bonds in the molecule, and/or no more than 4.0% of triglycerides with 3 residues of the same polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds.

35 18. An oil/fat or triglyceride containing at least 20% of arachidonic acid, and which contains at least 40%

of triglycerides with one residue of arachidonic acid in the molecule and/or no more than 4.0% of AAA.

19. An oil/fat or triglyceride containing at least 20% of dihomog- $\gamma$ -linolenic acid, and which contains at least 40% of triglycerides with one residue of dihomog- $\gamma$ -linolenic acid in the molecule and/or no more than 4.0% of DDD.

20. An oil/fat or triglyceride containing at least 20% of mead acid, and which contains at least 40% of triglycerides with one residue of mead acid in the molecule and/or no more than 4.0% of MMM.

21. A production process for a transesterified oil/fat or triglyceride, containing polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds and one or more medium-chain fatty acids as constituent fatty acids, by transesterification of 50 - 100 parts by weight of one or more fungus-produced oils/fats or triglyceride containing at least 20% of polyunsaturated fatty acids containing 20 or more carbons and two or more double bonds and 0 - 50 parts by weight of one or more medium-chain fatty acid triglycerides (ZZZ), using a 1,3-position specific type lipase.

22. A production process according to claim 21 wherein the polysaturated acid containing 20 or more carbons and two or more double bonds is arachidonic acid.

23. A production process according to claim 21 or 22 wherein the medium-chain fatty acid triglyceride is tri-octanoic acid glyceride.

24. A transesterified oil/fat or triglyceride obtained by a production process according to any one of claims 21 to 23.

25. A transesterified oil/fat or triglyceride containing at least 1 wt% of each of a triglyceride containing arachidonic acid (A) and medium-chain fatty acid (Z) as constituent fatty acid, ZAZ (a triglyceride with 2 residues of medium-chain fatty acid and one

residue of arachidonic acid, wherein arachidonic acid is bound to the position 2) and ZZA (a triglyceride with 2 residues of medium-chain fatty acid and one residue of arachidonic acid, wherein arachidonic acid is bound to the position 1 or 3).

26. A transesterified oil/fat according to claim 25 wherein the medium-chain fatty acid is octanoic acid.

27. A human nutritive composition comprising an oil/fat or triglyceride according to any one of claims 10 to 20 and 24 to 26.

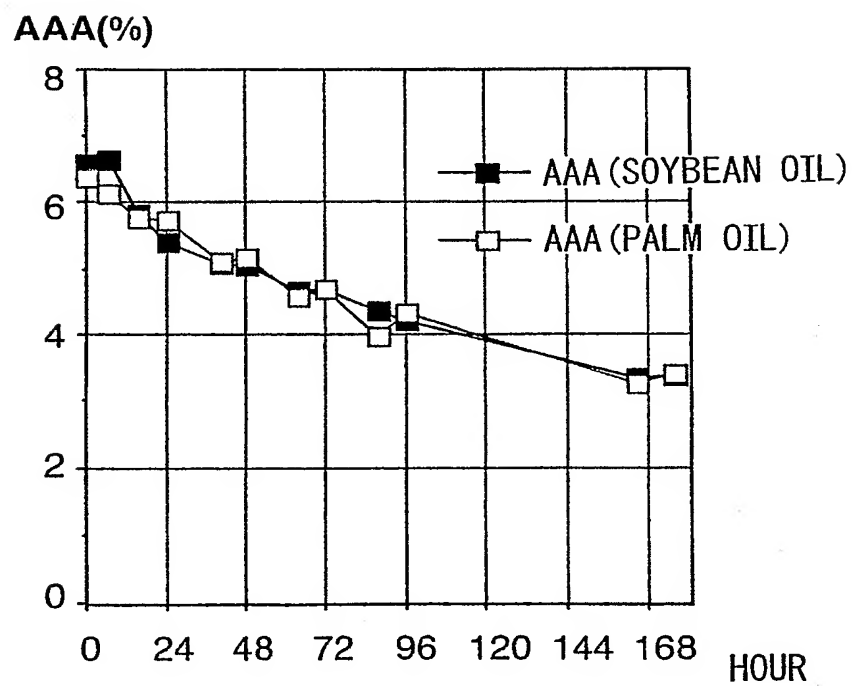
28. A food composition comprising an oil/fat or triglyceride according to any one of claims 10 to 20 and 24 to 26.

29. A food composition according to claim 28, characterized in that the food composition is a functional food, nutritional supplement food, modified milk for premature infants, modified milk for infants, infant food, maternal food or geriatric food.

30. An animal feed comprising an oil/fat or triglyceride according to any one of claims 10 to 20 and 24 to 26.

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Fig.1



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(54) Title: PROCESS FOR PRODUCTION OF TRANSESTERIFIED OILS/FATS OR TRIGLYCERIDES

(57) Abstract: There are provided polyunsaturated fatty acids-containing oils/fats or triglycerides with high digestion and absorption properties and resistance to oxidative damage, which are suitable for applications in fields such as modified milk for infants, food products and healthy foods and/or supplements, produced by transesterification of polyunsaturated fatty acids-containing oils/fats or triglycerides with vegetable oils/fats or triglycerides using 1,3-position specific type lipases.

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## INTERNATIONAL SEARCH REPORT

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## A. CLASSIFICATION OF SUBJECT MATTER

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

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☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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